



AMENDMENTS TO THE SPECIFICATION:

Please replace the paragraph at page 2 lines 9-26 with the following:

Some regenerative medicine treatments using stem cells or precursor cells derived from such cells have already been implemented, and infusion graft methods with hematopoietic stem cells or hematopoietic precursor cells are well known for treatment of conditions caused by a lack or functional deficiency of blood cells, such as leukemia and ~~a-plastic~~ aplastic anemia. However, stem cells present in parenchymal organs such as the brain, heart or liver are technically difficult to obtain from living tissues and/or to culture *in vitro*, and such stem cells also generally have low proliferation potency. Stem cells can also be recovered from tissues from corpses, but the medical use of cells obtained in this manner is associated with ethical problems. Consequently, regenerative treatments for neuropathy, cardiopathy and the like will require the development of techniques for generating desired cell types using cells other than stem cells present in such target tissues.

Please replace the paragraph at page 4 lines 24-34 with the following:

For reviews of ES cell properties and culturing methods, and their *in vivo* and *in vitro* differentiating abilities, refer to the following literature: Guide to Techniques in Mouse Development (Wasserman et al., Academic Press, 1993); Embryonic Stem Cell Differentiation in vitro (M.V. Wiles, Meth. Enzymol. 225:900, 1993); Manipulating the Mouse Embryo: ~~A Laboratory Manual~~ A Laboratory Manual (Hogan et al., Cold Spring Harbor Laboratory Press, 1994)(Non-patent document 1); Embryonic Stem Cells (Turksen, ed., Humana Press, 2002)(Non-patent document 2).

Please replace the paragraph at page 5 lines 28 to page 6 line 13 with the following:

When these pluripotent stem cells are used to produce research materials or regenerative medicine products, it is essential that the passaging methods used maintain the undifferentiated state and high proliferation potency of the cells. MEF cells or similar cells (such as STO cells) are usually used as feeder cells for ES/EG cells to maintain the undifferentiated state and high

proliferation potency of the cells. Addition of fetal bovine serum (hereinafter, FBS) to the culture medium is also important, and it is crucial to select an FBS product which is suited for the culturing of the ES/EG cells, usually with the addition of FBS at about 10-20%. Also, LIF has been identified as a factor that maintains the undifferentiated state of ES/EG cells derived from mouse embryo (Smith & Hooper, Dev. Biol. 121:1, 1987; Smith et al., Nature 336:688, 1988; Rathjen et al., Genes Dev. 4:2308, 1990), and addition of LIF to culture can more effectively maintain the undifferentiated state (see the following literature: Manipulating the Mouse Embryo: ~~A Laboratory Manual~~ A Laboratory Manual (Hogan et al., Cold Spring Harbor Laboratory Press, 1994 (Non-patent document 1) and Embryonic Stem Cells (Turksen ed., Humana Press, 2002)(Non-patent document 2)).

Please replace the paragraph at page 6 lines 14-35 with the following:

However, the culturing methods employed for these classical ES/EG cells are not suitable methods when human ES (or EG) cells are used for regenerative medicine or other practical purposes. One reason for this is that human ES cells are unresponsive to LIF, and lack of feeder cells causes death of the cells or loss of the undifferentiated state and differentiation into different cell types (Thomson et al., Science 282:1145, 1998). The use of feeder cells itself is another problem because [[as]] such co-culturing systems increase production cost and are poorly suited for large-scale culturing, while separation and purification of the ES cells from the feeder cells is required when the ES cells are to be actually used. In the future, when human ES cells and other pluripotent stem cells are utilized as cell sources for regenerative medicine, and particularly for cell transplantation therapy, the use of non-human animal cell products such as MEF cells and FBS will not be desirable because of risks including potential infection of the ES cells by heterozoic viruses and contamination with antigenic molecules that may be recognized as heteroantigens (Martin et al., Nature Med. 11:228, 2005).

Please replace the paragraph at page 12 lines 25-27 with the following:

Non-patent document 1: Manipulating the Mouse Embryo: ~~A Laboratory Manual~~ A Laboratory Manual (Hogan et al., Cold Spring Harbor Laboratory Press, 1994).

Please replace the paragraph at page 15 lines 31-36 with the following:

(2) A gene transfer method for pluripotent stem cells, characterized by efficiently transferring a gene into the pluripotent stem cells and ~~express~~ expressing it, using a liquid medium and a culturing vessel having immobilized or coated on a substrate solid phase surface a molecule which is adhesive to the pluripotent stem cells.

Please replace the paragraph at page 22 line 36 to page 23 line 20 with the following:

Unless otherwise specified, gene engineering methods employed in molecular biology and recombinant DNA technology, as well as common cell biology protocols and conventional techniques, may be employed for carrying out the invention, with reference to standard literature in the field. These include, for example, Molecular Cloning: ~~A Laboratory Manual~~ A Laboratory Manual, 3rd Edition (Sambrook & Russell, Cold Spring Harbor Laboratory Press, 2001); Current Protocols in Molecular Biology (Ausubel et al. ed., John Wiley & Sons, 1987); Methods in Enzymology Series (Academic Press); PCR Protocols: Methods in Molecular Biology (Bartlett & ~~Stirling~~ Stirling, eds., Humana Press, 2003); Animal Cell Culture: A Practical Approach, 3rd Edition (Masters ed., Oxford University Press, 2000); and Antibodies: ~~A Laboratory Manual~~ A Laboratory Manual (Harlow et al. & Lane ed., Cold Spring Harbor Laboratory Press, 1987). The reagents and kits used for the cell culturing and cell biology experiments referred to throughout the present specification are available from commercial vendors such as Sigma, Aldrich, Invitrogen/GIBCO, Clontech and Stratagene.

Please replace the paragraph at page 26 line 37 to page 27 line 32 with the following:

The present invention relates to a method of culturing pluripotent stem cells including ES cells and is characterized by using molecules that adhere to pluripotent stem cells (hereinafter referred to as "pluripotent stem cell-adhering molecules"). The pluripotent stem cell-adhering molecules used for carrying out the invention are used for the culturing method of the invention by being immobilized or coated on the solid phase surface of a culturing vessel or culture substrate

(hereinafter also collectively referred to culture substrate). Any culture substrate may be used as the culture substrate of the invention as long as it allows culturing of pluripotent stem cells, but preferably it is one used in the prior art for cell culturing. As examples of culture substrates for cell culturing there may be mentioned a dish, plate, flask, chamber slide, tube, cell factory, roller bottle, spinner flask, hollow fibers, microcarriers, beads and the like. These culture substrates may be made of inorganic materials such as glass, or of organic materials such as polystyrene, but it is preferable to ~~[[used]]~~ use materials such as polystyrene that have high adsorption properties for proteins and peptides, or materials that have been treated by, for example, hydrophilic treatment or hydrophobic treatment for increased adsorption properties. Also preferred are sterilizable materials with high heat resistance and moisture resistance. As an example of such a preferred substrate there may be mentioned a polystyrene dish and/or plate with no special cell culturing treatment (hereinafter referred to as "untreated polystyrene plate"), most commonly used for culturing of *E. coli* and the like, and such culture substrates are commercially available.

Please replace the paragraph at page 37 line 25 to page 38 line 7 with the following:

As explained above, the state of undifferentiation of pluripotent stem cells means that the pluripotent stem cells are capable of prolonged or virtually indefinite proliferation and exhibit normal karyotype (chromosomes), while having the capacity to differentiate into all three germ layers under the appropriate conditions. Also, they preferably have at least one of the other properties of pluripotent stem cells such as telomerase activity maintenance, teratoma formation, or ability to form chimeras. Methods of examining cell character and properties may be easily carried out using established standard protocols with reference to the literature cited above such as, for example, Guide to Techniques in Mouse Development (Wasserman et al. eds., Academic Press, 1993); Embryonic Stem Cell Differentiation in vitro (M.V. Wiles, Meth. Enzymol. 225:900, 1993); Manipulating the Mouse Embryo: ~~A Laboratory Manual~~ A Laboratory Manual (Hogan et al. eds., Cold Spring Harbor Laboratory Press, 1994); or Embryonic Stem Cells (Turksen ed., Humana Press, 2002), but there is no particular restriction to these methods.

Please replace the paragraph at page 41 lines 3 to 33 with the following:

As methods of recovering pluripotent stem cells there may be mentioned methods using publicly known enzyme treatment, which are ordinarily employed for passaging of pluripotent stem cells. As a specific example, there may be mentioned a method wherein the medium is removed from a culturing vessel in which pluripotent stem cells have been cultured, PBS is used for rinsing several times, preferably 2-3 times, a solution containing an appropriate protease (for example, a solution containing a protease such as trypsin or dispase) is added, culturing is carried out at 37°C for an appropriate period, preferably about 1-20 minutes and more preferably 3-10 minutes, and then the mixture is suspended in an appropriate solution such as the aforementioned ES cell culturing medium to obtain single cells. ~~Non-enzymatic~~ Non-enzymatic methods may also be used, and for example, there may be mentioned a method wherein the medium is removed from a culturing vessel in which pluripotent stem cells have been cultured, PBS is used for rinsing several times, preferably 2-3 times, an ethylenediamine tetraacetate (EDTA) solution is added to a final concentration of 0.01-100 mM, preferably 0.1-50 mM and more preferably 1-10 mM, for treatment at 37°C for an appropriate time, preferably about 1-60 minutes and more preferably 10-30 minutes for detachment of the cells, and then the mixture is suspended in an appropriate solution such as the aforementioned ES cell culturing medium to obtain individual cells. The same method may also be carried out using ethyleneglycol bis(2-aminoethylether)tetraacetate (EGTA) instead of EDTA.

Please replace the paragraph at page 42 line 35 to page 43 line 8 with the following:

The invention also relates to a method of generating a chimeric embryos or chimeric animals using pluripotent stem cells prepared by the method disclosed by the invention, and to the generated chimeric embryos and chimeric animals. Standard protocols have already been established for generating chimeric embryos and chimeric animals, and they can be easily generated with reference to, for example, Manipulating the Mouse Embryo: ~~A Laboratory Manual~~ A Laboratory Manual (Hogan et al. eds., Cold Spring Harbor Laboratory Press, 1994), though there is no particular limitation to this reference.

Please replace the paragraph at page 45 lines 8-26 with the following:

The ES cell lines used were EB3 cells (provided by Prof. Hitoshi Niwa of RIKEN), R1 cells (Nagy et al., Proc. Natl. Acad. Sci. USA 90:8424, 1993) and 129SV cells (obtained from Dainippon Pharmaceutical Co. Ltd.), and the experimental results showed no differences between the different ES cell lines. These ES cells were passaged according to the methods described in Manipulating the Mouse Embryo: ~~A Laboratory Manual~~ A Laboratory Manual (Hogan et al. eds., Cold Spring Harbor Laboratory Press, 1994) and Embryonic Stem Cells: Methods and Protocols (Turksen ed., Humana Press, 2002), using KnockOut-DMEM (Invitrogen) medium containing 10% FBS, 0.1 mM MEM non-essential amino acid solution, 2 mM L-glutamine and 0.1 mM 2-mercaptoethanol (hereinafter referred to as ESM), with addition of 1000 U/mL LIF (ESGRO; Chemicon), while maintaining their undifferentiated states, and they were supplied for experimentation. The ES cells passaged under these conditions will hereinafter be referred to as “ES cells passaged under ordinary conditions”.

Please replace the paragraphs at page 56 lines 11-36 with the following:

The teratoma-forming ability of the ES cells was examined next. A teratoma is a tumor, comprising fetal ~~tissue~~ and mature tissues from three germ layers of endoderm, mesoderm and ectoderm, which is formed when ES cells are transplanted into an animal such as a mouse, and teratoma-forming ability is used as an indicator of the pluripotency of ES cells.

ES cells (EB3 line) were seeded on a gelatin plate and an E-cad-Fc plate and passaged 5 times every three days. The ES cells were injected into Balb/c nude mouse ~~ovaries~~ testes (approximately 200 cells each) by an ordinary tests, and on day 60, teratoma formation was found in all of the ES cell-transplanted testes, with no noticeable difference in tumor size between the gelatin plate-cultured group and the E-cad-Fc plate-cultured group. Also, upon preparing tissue sections by a common method and observing the histology, the teratomas of both groups had ectodermal tissue/cell formation including epidermal-like tissue and neurons which were positive for different neuron markers (β III-tubulin, GFAP, neurofilament M, GAP-43), mesodermal tissue/cell formation including bone, cartilage and skeletal muscle-like tissue and endodermal tissue/cells including intestinal and bronchoepithelial-like tissue, and therefore

the ES cells cultured on E-cad-Fc plate were confirmed to have maintained teratoma-forming ability.

Please replace the paragraph at page 57 lines 3-19 with the following:

It was determined whether ES cells passaged multiple times on an E-cad-Fc plate retain chimera-forming ability. ES cells (EB3 cell line) taken from the same cell lot of frozen stock confirmed to have chimera-forming ability were seeded on a gelatin plate and E-cad-Fc plate, and were passaged 5 times every three days. The ES cells were injected into C57BL/6 mouse blastocysts (approximately 100 cells each) by an ordinary method, and these were transplanted into the uteruses of pseudopregnant ICR mice (8-10 weeks old) ~~uterus~~ and brought to parturition. C57BL/6 mice are normally black-haired, but some newborn individuals will have ES cell-derived agouti-colored hair on a portion of the body (5-80%); a total of four such chimeric mice were obtained from the ES cells cultured on a gelatin plate, and a total of seven were obtained from ES cells cultured on an E-cad-Fc plate.